Chapter 6

Dopaminergic receptor D_2 contribution on aggressive behavior: new insights about acute and chronic conditions

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Abstract

Introduction: Aggression is one of the most basic components of the limbic system, especially in social beings. Aggression is associated with the production and release of several neurotransmitters, and one tightly related to it is dopamine (DA). Binding of DA to dopamine receptor type 2 (D_2) receptors was suggested to be involved in pathological aggression in chronically aggressive humans and animals. The aim of this study was to evaluate changes in D_2 receptor availability with [^{11}C]-raclopride PET in a rat model of aggression. **Methodology:** Long-Evans rats from 2 independent studies with a repeated social defeat protocol (RSD) were used and screened for aggression. Controls and aggressive rats were stratified based on their attack latency during the training phase. Aggressive animals were further used as aggressors in a series of repeated social defeat trials. After RSD trials, the 2 cohorts of aggressive animals and unexposed non-aggressive controls were scanned for D_2 receptor availability using binding potential (BP_{ND}), as determined by dynamic [^{11}C]-raclopride PET scans. Caudate and putamen (striatum) and nucleus accumbens (NAc) binding potentials were calculated by kinetic modeling using a reference region (cerebellum). **Results:** Surprisingly, aggressive animals from cohort 1 showed a shorter attack latency in the period immediately before the PET scan, when compared aggressive animals from the second cohort (last trial: 42.84 ± 44.67s vs. 12.41 ± 13.60s; p=0.03). [^{11}C]-raclopride PET showed that these animals had also a significantly higher BP_{ND} in the striatum when compared with cohort 2 of aggressors ($p<0.001$) and the control group ($p<0.001$). However, there were no differences in BP_{ND} in the NAc between any of the groups. **Discussion:** Attack latency throughout several exposures is a parameter used to discriminate the level of aggression in animals. Our results for cohort 1 - but not for cohort 2 - agree with literature suggesting a relation between DA and aggressive behavior. The differences found in the D_2 receptor binding between cohort 1 and 2 of aggressive animals might be related with differences in their level of aggression at the time of the PET scan, which may be due to some experimental differences between the cohorts, such as the total number of RSD trials the aggressors were exposed to (29,4±4,9 vs 14,1±4,8 for cohorts 1 and 2, respectively), the frequency of the RSD trials (1.5 trial/week vs. 0.9 trial/week for cohort 1 and 2, respectively), or the interval between the last RSD trial and the PET scan (1 day and 14 days for cohort 1 and 2, respectively). To exclude the last parameter, the PET scan was repeated 4 weeks after the last RSD trial in cohort 2 (for practical reasons), but results were similar to those of the PET scan performed immediately after the last RSD trial. **Conclusion:** Our results suggest that aggressive behavior is associated with an increase in D_2 receptor availability in striatum, but not in NAc. The discrepancy between the 2 cohorts of aggressive animals in this study seems to be related to the aggressiveness of the animals at the time of the PET. This difference in aggressiveness between the cohorts may be due to overexposure of the animals of

cohort 2 to aggressive events, which could have blunted the rewarding reaction of the animals. Nonetheless, the results suggest a participation of D_2 receptors in aggressive behavior, which might, therefore, be a potential pharmacological target for diseases associated with aggressive events.

Keywords: aggressive behavior; dopamine; D_2 receptor; positron emission tomography; repeated social defeat

Introduction

Aggression is a functional behavior that is affected by a wide variety of experiences [1,2], such as possession of a resource [3], increase the likelihood of gene transmission over generations [4,5], and social dominance. Although aggression is important for the survival of an organism, and *per se* is considered normal behavior, excessive aggression and violence are associated with brain disorders, such as personality disorders, schizophrenia, depression, Alzheimer's disease, brain injury, and others [6–9]. Pathological aggression is the leading cause of all child and adolescent referrals to mental health clinicians [10,11], and therefore a serious concern for society. Aggressive behavior can be classified in two different subtypes: 1) controlled-instrumental, or the aggressive behavior needed in order to achieve a specific goal; and 2) reactive-impulsive, which is mostly driven by emotional behavior (e.g. anger). In humans, aggression can be seen as adaptive behavior, which can develop from reactive and instrumental to appetitive or rewarding aggression [12,13], which activates the reward circuitry in the brain, mimicking the effects of drugs. Indeed, appetitive aggression shows some core similarities with addiction, like the relapse (recidivism) rates between aggressive offenders and drug addicts, and the desire for aggressive-events despite short or long-term harmful consequences [14].

Molecular mechanisms involved in normal and abnormal aggression, including its relationship with dopaminergic (DA) transmission, have been studied in several species. Literature suggests that dopamine release is associated aggressive behavior [15–17], an idea which is supported mostly by pharmacological studies showing that drugs that modulate dopaminergic transmission are able to contain aggressive-like behavior [18–20]. Additional studies using positron emission tomography (PET) have also shown the relationship between aggression and dopamine synthesis and dopamine receptor D_1 availability [21–26]. PET imaging studies on aggression mainly reflect the chronic disposition of dopamine concentration in patients who have shown recurrent aggressive behavior, with no data regarding the acute effect of an aggressive episode on dopamine levels. Understanding the contribution of the dopaminergic system to aggression can lead to alternative treatments for people suffering from pathologies associated with aggressive behavior. Additionally, understanding how brain dopamine release and binding to its receptors is changed after acute aggressive life events can contribute to better understand how normal aggression becomes pathological, thus helping in the prevention of this kind of antisocial behavior. In this regard, dopamine D_2 receptors are associated with the addiction pathway and can be a possible factor of turning aggressive behavior into a more pathological state [27,28].

Therefore, the aim of this study was to evaluate how dopaminergic D_2 receptors behave in animals showing aggressive behavior using [^{11}C]-raclopride PET imaging. To achieve this, animals

were trained using the repeated social defeat protocol to develop appetitive aggression through repetitive winning confrontations [29] and divided into groups according to their aggression level (aggressors or controls). Both controls and aggressors were scanned for D_2 receptor availability.

Materials and Methods

Subjects

Outbred 16-weeks old male Long Evans (LE) (Harlan, Indianapolis) from 2 studies (6828B and 171706-01-004) undergoing the same repeated social defeat protocol were studied. The animals were divided into 3 groups: Non-aggressive controls (n=6), aggressive animals from study 171706-01-004 (cohort 1, n=13) and aggressive animals from study 6828B (cohort 2, n=12).

One week after arrival, the male LE rats were paired with tubal-ligated LE female rats in a large cage (80x50x40cm), with *ad libitum* access to water and food. Animals were maintained in a controlled room with a 12:12 hour light/dark cycle (lights on at 7 p.m.), regulated temperature (21±2°C) and humidity controlled environment. Male and female LE rats were paired and housed together at least one week prior to the screening phase of experiment in order to increase male territoriality. All animal experiments were performed according to the Dutch Law for Animal Welfare and were approved by the Institutional Animal Care and Use Committee of the University of Groningen, under protocols 171706-01-001 and 171706-01-004.

Study design

The study was conducted in 3 different stages (see Figure 1). In the first stage, LE males were trained and screened for aggression during 5 consecutive days. During the screening sessions, females were removed from the cages 1 hour before an intruder was introduced inside the resident's cage. To avoid habituation, each day of aggressive trial consisted of a different intruder being presented to the resident. Time measurements for the latency to attack of the resident and the time of successful submission (i.e. when intruders submitted for more than 3 consecutive seconds) were performed. After intruders submitted, or after 10 minutes if they did not, intruders and residents were separated by a barrier placed inside the resident's cage. Sixty minutes after introduction, the intruders were removed from the resident's cage.

Residents were classified according to their average attack latency over the 5 consecutive screening sessions as follows: not aggressive (more than 60 seconds), aggressive (between 10 and 60 seconds), and violent (less than 10 seconds, accompanied with violent behavior, i.e. direct attack to

vital zones of the body, no threatening before attacking [29]). Violent animals were excluded. After this selection, aggressive animals were used for the second stage, consisting on several trials of repeated social defeat (RSD) over six months (cohort 1: 14,1±4,8; cohort 2: 29,4±4,9). On the third stage, aggressive animals and controls were submitted to a dynamic [^{11}C]-raclopride PET scan either 1 day (cohort 2) or 2 weeks (cohort 1) after the last exposure to a resident-intruder paradigm. In cohort 2, the PET scan was repeated after 4 weeks to exclude any time interval effects.

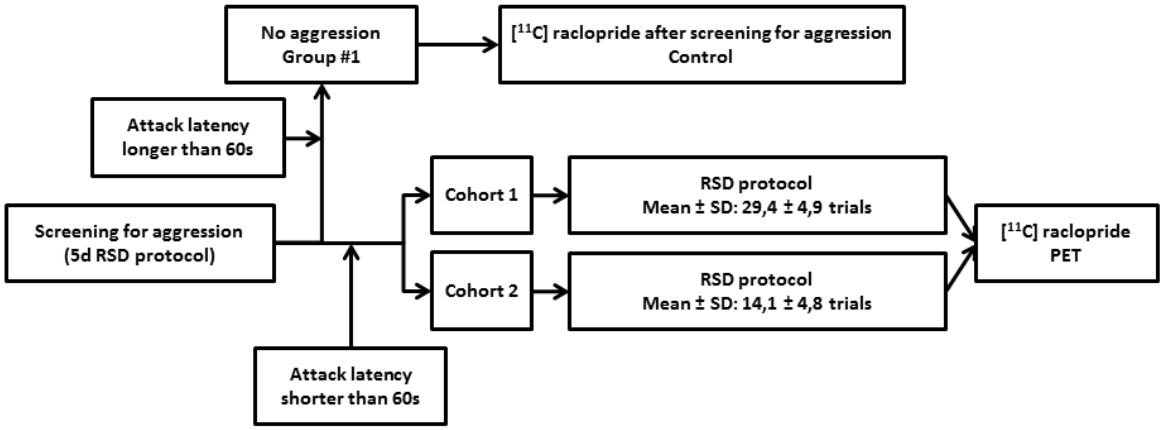

Figure 1: Study development protocol. Abbreviations: RSD: Repeated social defeat; PET: Positron emission tomography imaging.

Repeated social defeat (RSD)

This protocol was performed as described elsewhere [29,30] Wistar rat intruders of smaller size were introduced into the resident's cage, and after the residents explored the intruder and threatened them, latency to attack and latency of submission was measured. The submission was achieved when the intruder assumed a supine (submissive) position for at least 3 seconds. Animals were allowed to fight until the intruder was submitted, or when the maximum time of 10 minutes since the introduction of the Wistar animals was reached. Either after submission or 10 minutes of the encounter, intruders and residents were separated by a physical barrier in the resident cage that allows visual, auditory and olfactory (but not physical) contact until a period of 60 minutes since the first introduction was complete. After completion of the 60 minutes period, animals were returned to their own cages and females placed back into the resident cages. All RSD experiments were performed between 14:00 and 18:00 hrs.

Radiotracer preparation and dynamic PET imaging.

Brain imaging of D_2 receptors was performed using the radiotracer [^{11}C]-raclopride. The synthesis of the radiotracer was performed through alkylation of S-(+)-O-desmethylraclopride (ABX, Radeberg, Germany) using ^{11}C-methyl triflate, as described previously [31]. PET scans were performed using a small animal PET scanner (Focus 220, Siemens Medical Solutions, USA). Prior to PET imaging, animals were anesthetized using a mixture of isoflurane and oxygen (5% for induction, 2% for maintenance) and a tail vein cannula was placed for tracer injection. Heating pads were used to maintain body temperature throughout the experiment. Before tracer injection, animals were placed in the PET camera in prone position with their head in the field of view. A transmission scan was performed using a ^{57}Co point source for attenuation and scatter correction. [^{11}C] raclopride was injected (25.75±9.3 MBq) over 1 minute using an automatic injection pump at a speed of 1 mL/min, at the same time the acquisition of the 60 minutes dynamic PET scan was started. During the scan, temperature, heart rate, and blood oxygen saturation were monitored.

Image processing and PET analysis

The 60 min emission list-mode data was used to reconstruct the image in 21 frames (6 x 10s, 4 x 30s, 2 x 60s, 1 x 120s, 1 x 180s, 4 x 300s and 3 x 600s). The frames were iteratively reconstructed using an attenuation-weighted two-dimensional ordered-subset expectation maximization algorithm (OSEM2; 4 iterations and 16 subsets), and corrected for random coincidences, scatter, decay and attenuation. Final images had a 128 x 128 x 95 matrix with a pixel width of 0.475 mm and slice thickness of 0.796 mm. The PET images were individually co-registered to a [^{11}C]-raclopride brain template [32] (PMOD 3.8; PMOD Technologies LLC, Switzerland) which allowed the use of a predefined volume-of-interest (VOI) map and the reporting of results in Paxinos stereotactic coordinates from the rat brain. After co-registration, time-activity curves (TACs) were generated for nucleus accumbens core and shell (NAc), caudate and putamen (striatum) and cerebellum by applying a predefined mask of VOIs to the dynamic data, as stated elsewhere [32]. Due to limited resolution of the PET scanner used (1.4 mm), only the whole NAc and striatum – and not subsections thereof - have been assessed in order to obtain a better signal-to-noise ratio. The simplified reference tissue model (SRTM) with cerebellum as a reference region was used to quantify tracer uptake [31,33]. The non-displaceable binding potential (BP_{ND}) was calculated for the chosen areas using the cerebellum as a reference.

Statistical analysis

All reported data are shown as mean ± SEM. Data were analyzed using IBM SPSS 23 software (IBM; Armonk, NY). PET data was analyzed with a one-way analysis of variance (ANOVA) and linear regression was performed to assess effect of number of trials on attack latency. For all differences, a $p<0.05$ was considered as statistically significant.

Results

Aggressive behavior is altered between groups

Analysis of aggressive behavior by linear regression showed that aggressive animals from cohort 1 had increasingly shorter attack latency until the last trial ($R^2=0.68$, $p<0.001$), with a decrease of 1.7 seconds per exposure. When looking at the first ten trials only, both cohorts show a significant correlation between attack latency and the trial number (Cohort 2: $R^2=0.632$, $p=0.005$; cohort 1: $R^2=0.49$, $p=0.022$ – Figure 3A). However, when observing the remaining trials, the significant correlation is maintained only in cohort 1, although the statistical significance is marginal ($R^2=0.41$, $p=0.046$). In cohort 2, there is no correlation at all between attack latency and trial number for trials 11-38 (figure 3B). Consequently, the average attack latency of the last 10 trials before the PET scan was significantly shorter for cohort 1 than cohort 2 (17.41 ± 3.234 vs. 32.89 ± 4.124; $p=0.008$)

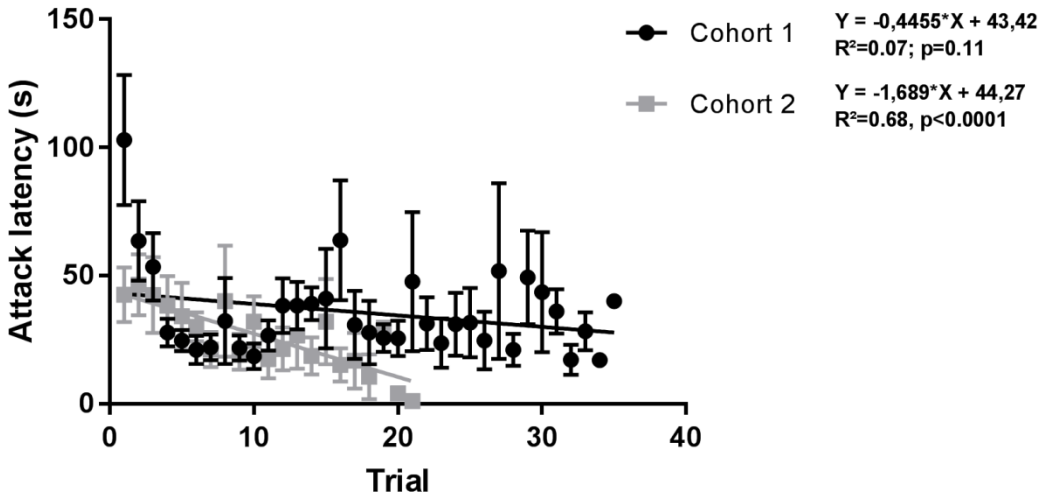

Figure 2: Linear relation of attack latency with trials (time) in both cohorts of animals. Lines represent the linear trend of each group. The equation represents curve slope.

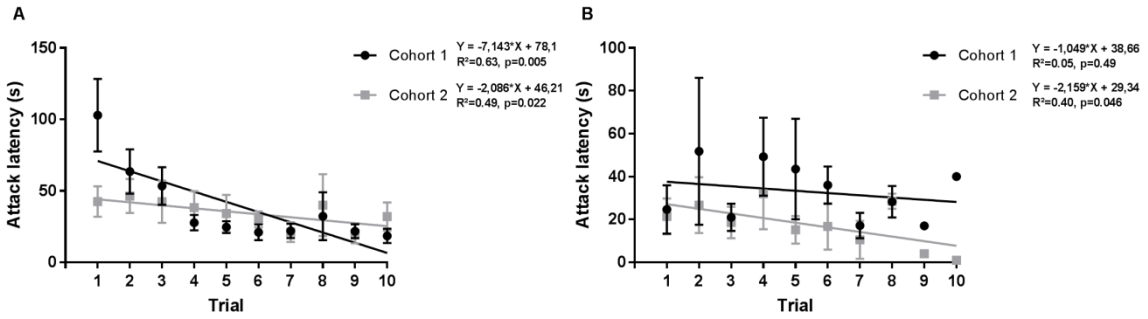

Figure 3: Linear relation of attack latency with the first 10 trials (A) and the remaining trials (B) in both cohorts of aggressive animals. Lines represent the linear trend of each group. Numbers on the X-axis of B are not equivalent to trial numbers. The equation represents curve slope.

Striatal D_2R binding potential is altered by interval from last aggression

[^{11}C]-raclopride PET showed a significant difference in the BP_{ND} in the striatum of aggressive animals from cohort 1 when compared with both controls and aggressive animals for cohort 2 ($F_{(2,25)}$=26.041; p<0.001). There was no significant difference between aggressor animals from cohort 2 and the control group (p>0.05). Repetition of the PET scan in cohort 2 four weeks after the last RSD trial revealed similar [^{11}C]-raclopride binding in striatum as compared to the PET scan made 1 day after the last RSD trial (data not shown).

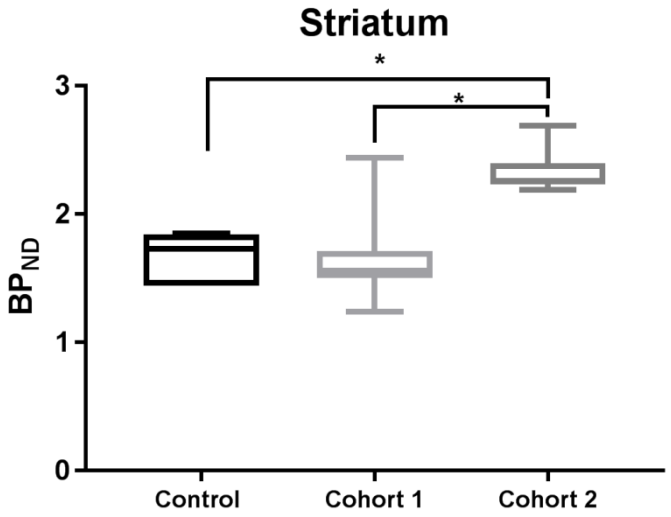

Figure 4: Effect of aggression on [^{11}C]-raclopride binding potential in the striatum. Data expressed as median and interquartile range, whiskers represent the minimum and maximum values. *: p<0.05. N 7-11 per group.

In the nucleus accumbens, there was no significant difference in the binding potential of [^{11}C]-raclopride between groups (all p>0.05). There was also no significant relationship between the uptake of [^{11}C]-raclopride in both brain regions assessed and the average attack latency (all p>0.05).

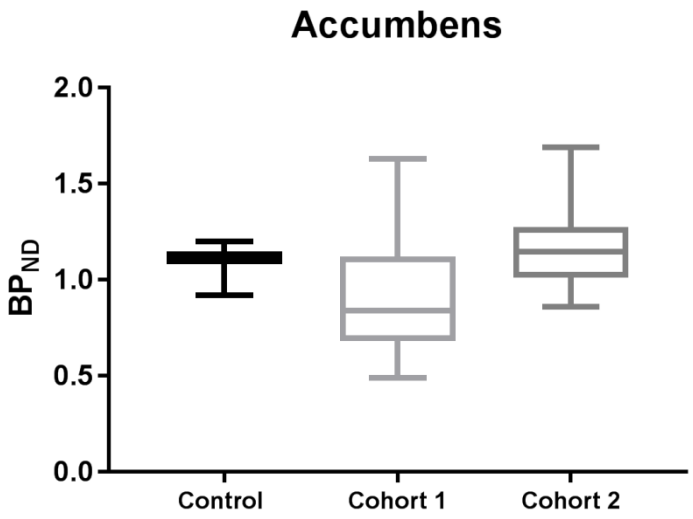

Figure 5: No effect of aggression on the [^{11}C]-raclopride binding potential in the nucleus accumbens. Data expressed as median and interquartile range, whiskers represent the minimum and maximum values. N: 7-11 per group.

Discussion

In the present study, we have shown changes in D_2-receptor availability in the striatum, but not in the nucleus accumbens, in one cohort of aggressive animals, but not in the other cohort. Additionally, the cohort of aggressors with increased availability of D_2 receptors showed a shorter attack latency than the other cohort of aggressive animals. These results suggest that changes in D_2 receptor availability could be linked to the aggressiveness of the animals. Changes in DA transmission due to aggression are not surprising. Our results are in agreement with literature showing a relation of DA release with aggressive and violent episodes in people with and without a diagnose of brain disorders [34,35], and also in animal models of aggressive behavior [15,36,37]. The observed increase in DA D_2 receptor availability is in line with the findings of Jupp and colleagues, who showed an increase of D_2/D_3 radiotracer binding in striatum using autoradiography. Nevertheless, they also found an increase in the nucleus accumbens, which was not observed in our study [38]. Increases in D_2 radiotracer binding in the accumbens was also observed by other studies in animal models of aggression [39–41]. Lack of differences in D_2 binding on our experiments may be explained by the limited spatial resolution of the PET camera used, thus making assessment of smaller brain regions such as the accumbens challenging. This generates an additional concern as the core and shell of the nucleus accumbens are shown to have distinct functionalities in the limbic system [42]. Future studies that aim to asses D_2 receptors in such small regions should take the spatial resolution of the assessment tool into account when performing data collection, and using other techniques such as immunohistochemistry might complement possible PET findings.

Animals that went through less aggressive bouts (cohort 1) showed an increased [^{11}C]-raclopride BP_{ND}, meaning that less dopamine is reaching the D_2 receptors in the striatum. The D_2 receptor is associated with inhibition of glutamate signaling in striatal medium spiny neurons (MSN), the main source of GABA [43]. Lack of dopamine reaching the D_2 receptors can lead to a downregulation of MSN activity and a decrease in GABA signaling towards the main limbic structures, such as the frontal cortex, amygdala, and ventral tegmental area. D_2 receptor-mediated signaling has been shown as a suppressing factor of aggression [44]. The PET results may be also explained by the interplay between D_2 receptor availability and DA release. Competitive PET tracers for neurotransmitter receptors are unable to distinguish if changes in BP_{ND} are due to an increased number of receptors, or an increase of endogenous dopamine [45]. In order to confirm this D_2 effect in aggression, it might be of interest for future studies to replicate the RSD protocol using animals with transiently impaired D_2 signaling (e.g. pharmacological intervention with raclopride prior to RSD trial).

In this study, attack latency was the parameter used to discriminate the level of aggression of animals [46]. Changes of dopaminergic receptor availability and/or dopaminergic transmission in different brain areas seem to be associated with the degree of aggression [35,39,41]. We found that the aggressive animals followed an expected pattern of aggression during the RSD, taking fewer seconds to initiate an attack during each session. Initially, the animals showed a gradual decreased in the attack latency with increasing number of exposures. In both cohorts of animals, this decrease in attack latency was observed until a certain point. In cohort 2, the attack latency remained relatively stable over time from this point onward. It is possible that the animals of cohort 2 became habituated to the protocol after a certain period of time, thus explaining why after a certain period of time the attack latency of the animals remains mostly unchanged. To the best of our knowledge, there are no studies regarding the effect that the number of exposures to RSD has on the aggressors, as these animals are not usually the main focus of studies involving this protocol, and usually are only briefly mentioned in reports. From our results, it is possible to assume that the aggressive behavior initially increases, but stabilizes after a certain point if the animals are overloaded with exposures to RSD. Future studies using the RSD protocol should, therefore, take into consideration the number of trials each animal goes through when planning the study.

However, the fact that animals of cohort 2 showed habituation to the protocol does not explain why the other cohort of aggressors showed a constant decrease in attack latency time until the end. One possibility is that these animals had a decreased burden, having a lower frequency of bouts of RSD during the protocol (14 vs 29 on average over 6 months) over the period of use of these

animals. Thus, animals of cohort 1 had no habituation due to a seemingly longer period between trials; or that animals of cohort 2 had so higher a burden that they showed exhaustion towards the end of the protocol.

In conclusion, our results showed a gradual reduction in attack latency over time in both groups at the beginning of the experiment, but this effect was lost after longer periods in the cohort with a higher number and higher frequency of exposures. This might be due to habituation or due to exhaustion after several RSD trials. We also found an increase of D_2 tracer binding in the striatum of the more aggressive rats in cohort 1, but not in the less aggressive cohort 2. Additional experiments like immunohistochemistry, microdialysis or liquid chromatography to assess DA itself are needed to discriminate if those results are due to differences in DA release, or due to differences in receptor expression. Nonetheless, these results suggest the participation of D_2 receptor-mediated signaling in aggressive behavior, which can be used as a pharmacological target for diseases associated with aggressive events.

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